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Receptor-Mediated Endocytosis

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<b>13. ABSTRACT (Maximum 200 Words)</b>  My proposal has envisioned a study of endocytic rate of EGFR in cells expressing different versions of erbB2. ErbB receptors are dimerized by their ligands, leading to receptor activation and internalization. Internalized receptors are degraded in lysosome, terminating the signal. The C-terminal region of EGFR contains endocytic motifs that regulate the internalization of EGFR. ErbB2 may also contain endocytic motifs. ErbB2 dimerizes with EGFR, and it may regulate the endocytosis rate of its dimerization partner. The presence of erbB2 may retard the internalization of EGFR, prolonging its presence on cell surface and potentiating its activation. I was able to show in transiently transfected HeLa cells that transient overexpression of erbB2 led to a decrease of endocytosis, measured indirectly by phosphorylation of hrs1. I was unable to develop a stable erbB2 overexpressing line, however. Several studies since the submission of my proposal supported the idea that erbB2 expression leads to a decrease in EGFR internalization (Wang et al., 1999; Worthylake et al., 1999). Another study showed that erbB2 overexpression in BT-20 cells led to prolonged downstream signaling by Raf-1 and MAPK, compared with cells that expressed lower level of erbB2 (Zhang et al., 2002). These studies provided support to my hypothesis that erbB2 plays a regulatory role on EGFR signaling by modulating its internalization.				
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## Introduction

The premise of my project, I believe, remains valid. The level of EGF receptor (EGFR) on cell surface is regulated by receptor internalization and recycling. Receptors are internalized following their activation, and some of the internalized receptors are directed to lysosomes, where they are degraded. Overexpression of EGFR on cell surfaces has been linked to breast cancers, underscoring the importance of regulation of EGFR level on cell surface. Dimerization of erbB receptors is necessary for their activation. For the family of erbB receptors, which includes EGFR, it is thought that dimerization between either the same erbB receptors or different ones leads to their activation. EGFR forms dimers with each of the other three members of erbB family. Among them is erbB2, the overexpression of which has also been linked to breast cancers. ErbB2 has an intrinsically slower rate of internalization than EGFR. The difference in erbB receptors' ability to undergo internalization is believed to be regulated by the C-terminal sequences on the receptors. Based on these facts, I proposed that erbB2 dimerizes with EGFR, and the resulting dimers are impaired in receptor internalization. This would prolong the presence of activated EGFR on cell surface, resulting in increased EGFR signaling. I wanted to study the role of erbB2 in the regulation of internalization of EGFR, focusing on erbB2's C-terminal sequence. I wanted to know if manipulation of the C-terminal sequence by introducing mutation would alter the internalization and signaling of EGFR. Due to unexpected technical difficulties in setting up an internalization assay and established cell lines, I had not been able to make enough progress to be in a position to test the effects of mutations.

## Body

As I stated in my last report, I was working on an assay for EGF internalization. I originally planned to use an ELISA assay developed by Sandra Schmid and colleagues (Smythe *et al.*, 1992). Unfortunately, the antibody that she employed in the assay was a commercial antibody that was no longer available. A search for a replacement was not successful. Instead, I decided to develop an alternative assay. This assay utilizes a marker that monitors EGF internalization indirectly. Hrs-1 (Hepatocyte growth factor regulated substrate 1) is an endosomal protein that is tyrosine-phosphorylated only when EGF reaches the endosomes; in other words, only when EGF is internalized. Urbe and colleagues (2000) had shown that phosphorylation on hrs-1 is dependent on endocytosis and colocalization of EGF/EGFR with hrs-1 in early endosomes, to which internalized EGFR and its ligand are initially directed. I tested the assay with HeLa cells overexpressing the mutant dynamin, K44A. Dynamin is a molecule that is necessary for many forms of endocytosis that occur in cells. K44A is a dominant negative inhibitor of endocytosis because it blocks endocytosis in cells expressing normal dynamin. For control, cells overexpressing the wild type dynamin were also tested. As expected, in cells overexpressing the wild type dynamin, hrs-1 was strongly phosphorylated (Figure 1). On the other hand, in cells overexpressing K44A, phosphorylation on hrs-1 was substantially reduced. The residual phosphorylation was probably due to incomplete inhibition of endocytosis by K44A. I then tested my hypothesis that expression of erbB2 inhibits endocytosis of EGFR. For this test, HeLa cells were either transfected or not transfected with erbB2, and the hrs-1 assay was performed in both cases. Because a stably transfected cell line was not available, cells were transiently transfected. The

results of the assay supported my hypothesis (not shown, but a similar experiment was shown in Figure 4). Hrs-1 from cells not transfected with erbB2 was strongly phosphorylated, as expected, but hrs-1 from cells that were transfected with erbB2 showed very little phosphorylation. Western blot confirmed the overexpression of erbB2 in transfected cells, compared with untransfected parental cells.

The limitations of this assay are that it is an indirect assay, and it requires the presence of hrs-1 in the cells. If cells do not express hrs-1 at a sufficient level so that it can be detected in a Western blot, the assay is not applicable. Even in cells that express hrs-1, the expression level of the protein will determine how useful the assay is. To test this assay in a different cell type, I transfected MDA-MB 468, a human breast adenocarcinoma cell line, with erbB2. As shown in Figure 2, the parental cell line expressed little erbB2. In contrast, two MDA-MB 468 lines stably transfected with erbB2 expressed the protein at relatively high levels. Both parental and transfected lines expressed similar amount of EGFR. The hrs-1 assay was performed on both the parental line and erbB2-expressors. Judging from the results, it appeared that hrs-1 was expressed in MDA-MB 468, but at a lower level than in HeLa cells. In the parental cell line, hrs-1 was phosphorylated, though not impressively. In the erbB2 expressors, the phosphorylation on hrs-1 was reduced relatively to the parental line (Figure 3). Although there was a difference, it was not a decisive difference. Thus, one could not conclude with confidence that the erbB2 overexpression in MDA-MB 468 cell inhibited EGFR internalization, although this appeared to be the case.

A potential solution that could allow the assay to be used for cells that express low level of hrs-1 is to introduce a recombinant form of hrs-1. Kitamura and colleagues

(Komada et al., 1997) generated a HA-tagged hrs-1 for their study on hrs-1 localization. Urbe and colleagues used this construct to show the link between hrs-1 phosphorylation and EGF internalization. I obtained this construct from Dr. Kitamura. I transiently transfected HeLa cells with the HA-tagged hrs-1, and subjected the transfected cells to the hrs-1 assay. In addition using hrs-1 antibody to immunoprecipitate the protein, the anti-HA antibody was also used in a parallel set of experiments, so that only the exogenously introduced HA-hrs-1 would be immunoprecipitated. For positive control, immunoprecipitation was performed with anti-hrs-1. Surprisingly, the assay did not seem to work as well with HA-hrs-1 (Figure 4). Even though HA-hrs-1 was expressed, very little phosphorylation was detected on HA-hrs-1. The control experiment, on the other hand, worked as expected. I do not have an explanation why the HA-hrs-1 was not phosphorylated as well as the endogenous protein. Urbe and colleagues had shown in their paper that HA-hrs-1 was phosphorylated following EGF internalization, but in my hand it appeared phosphorylation was limited to the endogenous protein.

In addition to working on the assay, I had also devoted considerable time to establish HeLa cell lines stably expressing erbB2. The reason I wanted to develop HeLa cell lines expressing erbB2 was because I wanted to do the same experiments that Vieira and colleagues (1996) did to study the signaling properties of HeLa cells that were transfected with different forms of dynamins. Stable cell lines are useful because they express the protein of interest at constant levels, and having different stables available allows one to compare signaling at different levels of protein expression. I did not get very far at developing stable cell lines overexpressing erbB2, however. Although I obtained several cell lines expressing moderately higher amount of erbB2, relative to the

parental cell line, I had not been able to get a cell line that overexpress erbB2, for reasons that were not clear. The goal of the project was to study the signaling properties of EGFR in the presence of erbB2, and to determine if the C-terminal sequence of erbB2 affects the endocytosis and signaling of EGFR in an EGFR-erbB2 heterodimer. I wanted an erbB2-overexpressing cell line because it is important to establish that erbB2 overexpression does alter EGFR signaling. Without an erbB2-overexpressing line, I do not know whether or not erbB2 would alter EGFR signaling. Because it was not known whether or not it would be worthwhile to mutate erbB2, the mutations were not made.



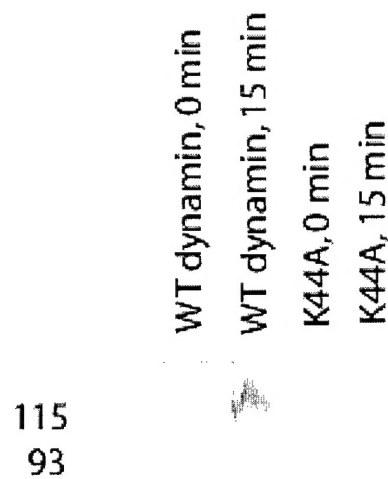


Figure 1. Hrs-1 assay for WT- and K44A-expressing HeLa cells. Cells were incubated with EGF on ice and shifted to 37 C for the specified amount of time. Hrs-1 was immunoprecipitated with hrs-1 antibody. PY20 (anti-phosphotyrosine) antibody was used for detect phosphorylation on hrs-1.

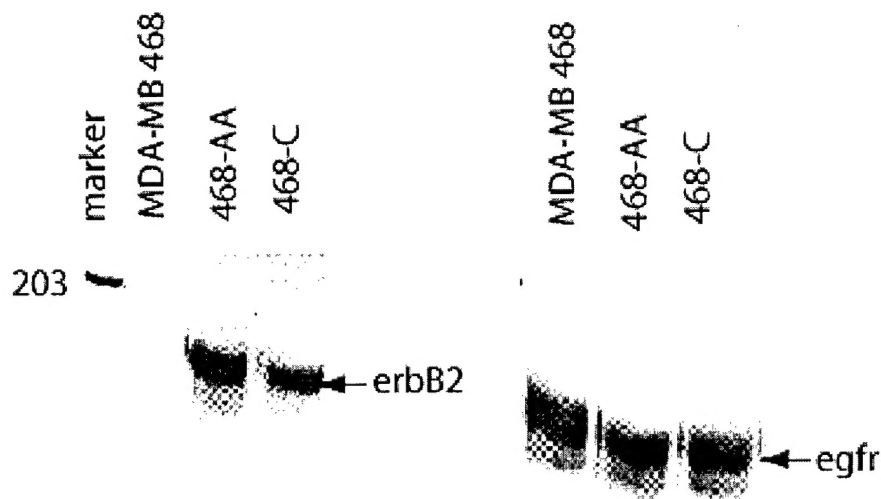


Figure 2. (left) Expression of erbB2 in MDA-MB 468 and two erbB2-overexpressing stable clones (468-AA and 468-C). (right) Expression of endogenous EGFR in the three lines.

hrs-1

MDA-MB 468, 0 min
468-AA, 0 min
468-C, 0 min
MDA-MB 468, 15 min
468-AA, 15 min
468-C, 15 min

Figure 3. Hrs-1 assay for MDA-MB 468 and erbB2-expressing 468 clones (468-AA and 468-C). Cells were incubated with EGF on ice, and shifted to 37 C for specified amount of time. Anti-hrs-1 was used for immunoprecipitation and PY20 was used to detect phosphorylation on hrs-1.

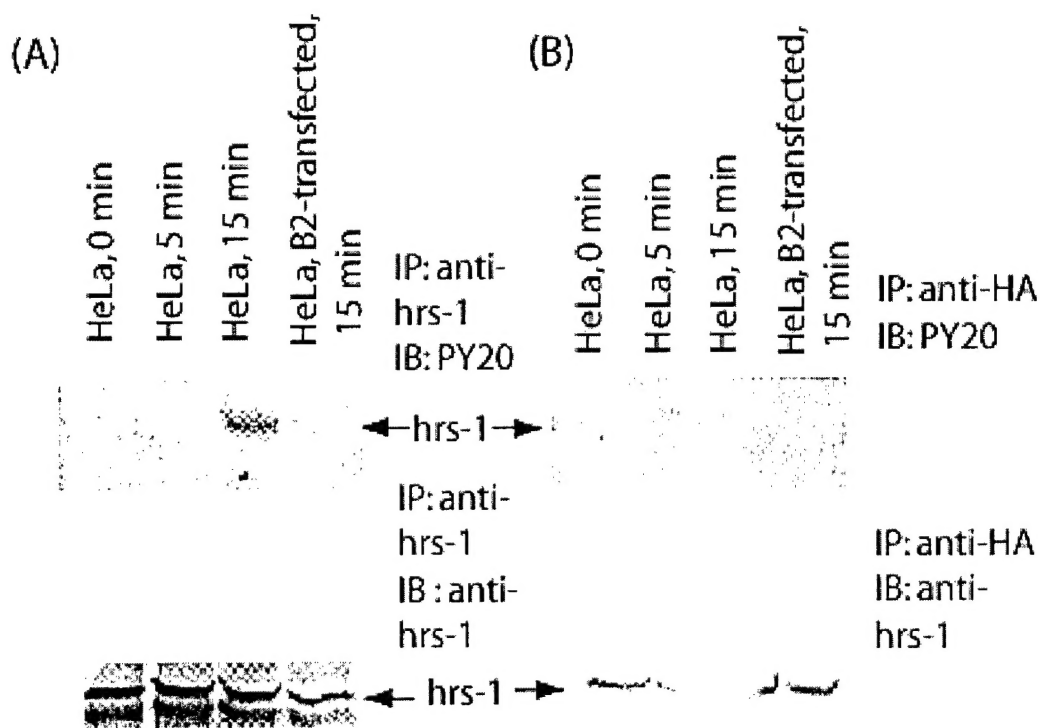


Figure 4. Hrs-1 assay for HeLa and erbB2(B2)-transfected HeLa. All were transfected with HA-hrs-1. Cells were incubated with EGF on ice, and shifted to 37 C for specified amount of time. (A) Hrs-1 was immunoprecipitated with anti-hrs-1, run on gel, and blotted with either anti-phosphotyrosine antibody (PY20, top) or anti-hrs-1 (bottom). (B) Same as (A), except anti-HA was used for immunoprecipitation.

### **Key Research Accomplishments**

1. Development of an endocytosis assay.
2. Development of erbB2 overexpressing line in MDA-MB 468 cell line.

### **Reportable Outcomes**

None.

## Conclusions

Ultimately, the project stalled because of two things: (1) delays in the development of an endocytosis assay, and (2) difficulties in generating a stable erbB2-overexpressing line in HeLa. The project was based on the Vieira et al. (1996) paper that showed endocytosis regulates EGFR signaling. It was originally envisioned that the ELISA assay developed by Sandra Schmid and colleagues would serve as a simple and quantitative assay of endocytosis. The unavailability of the key capture antibody, and my inability to find a replacement, forced me to turn to a different assay. I also had unexpected difficulties in generating an erbB2-overexpressing line in HeLa. Since the premise of the project was that erbB2 co-expression regulates EGFR internalization and signaling, it is important for me to establish that erbB2 overexpression does alter EGFR internalization and signaling. An erbB2-overexpressing line would allow me to investigate if different aspects of EGFR signaling are altered, and would serve as a basis of comparison for any future erbB2 mutants. Without such an overexpressing line, I could not verify the premise of the project, and because I did not know if it was worthwhile and useful for me to create erbB2 mutants, the mutants were not made.

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